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SO VETERINARY PARASITOLOGY, (MAY 1993) Vol. 47, No. 3-4, pp. 225-233.

SO Z PARASITENKD, (1983) 69 (1), 27-34.*

SO Revista Brasileira de Parasitologia Veterinaria, (1995) Vol. 4, No. 1, pp.* 15-19.

SO Vet.Rec. (111, No. 18, 414-15, 1982) 2 Fig. 2 Tab. 12 Ref.*

SO J PROTOZOOL, (1987) 34 (4), 398-402.

SO AM J TROP MED HYG, (1987) 36 (3), 505-508*

Applied and Environmental Microbiology, (1995) Vol. 61, No. 2, pp.*

No 3

SO Journal of Parasitology, (Feb., 1998) Vol. 84, No. 1, pp. 8-15.

SO Journal of the Egyptian Society of Parasitology, (Aug., 1999) Vol. 29, No. 2, pp. 551-560. *

SO FEMS Microbiology Letters, (1994) Vol. 118, No. 1-2, pp. 45-49.

SO J.Protozool. (35, No. 4, 583-89, 1988) 7 Fig. 2 Tab. 20 Ref. *

SO AM J TROP MED HYG, (1987) 36 (3), 505-508. *

SO J PARASITOL, (1976) 62 (2), 199-202.

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CONTENTS

from the other highly ciliates, such as polyhymenophorans, possessing an adoral zone of membranelles.

On the phylogenetic relationship between the entodiniomorphs having cilia on their vestibular walls, such as ophryoscolecids (3–5, 7, 10), and those having no cilia on it, such as *Cycloposthium*, the acquisition of cilia on the vestibular wall may be a result of functional differentiation for sending food more efficiently into the cytostome and assumes that the ophryoscolecids are a more differentiated group than cycloposthiids.

LITERATURE CITED

1. Corliss, J. O. 1979. *The Ciliated Protozoa. Characterization, Classification and Guide to the Literature,* 2nd ed. Pergamon Press, Oxford.

2. Fernández-Galiano, D. 1958. La infraciliación en *Cycloposthium edentatum* Strelkow. *Biol. Real. Soc. Esp. Hist. Nat.,* **57**: 139–150.

3. Fernández-Galiano, T., Serrano, S. & Fernández-Galiano, D. 1985. General morphology and stomatogenesis of two species of the genus *Entodinium* (Ciliophora, Entodiniomorphida). *Acta Protozool.,* **24**: 181–186.

4. Furness, D. N. & Butler, R. D. 1985. The cytology of sheep rumen ciliates. II. Ultrastructure of *Eudiplodinium maggii*. *J. Protozool.,* **32**: 205–214.

5. ——— 1985. The cytology of sheep ruman ciliates. III. Ultrastructure of the genus *Entodinium* (Stein). *J. Protozool.,* **32**: 699–707.

6. Hollande, A. & Batisse, A. 1959. Contribution à l'étude des infusoires parasites du coecum de l'hydrocheire (*Hydrocheirus capybara* L.). 1. La fammile des Cycloposthiidae. *Mem. Inst. Oswaldo Cruz Rio de J.,* **57**: 1–16.

7. Imai, S., Tashiro, H. & Ishii, T. 1983. Scanning electron microscopy of the adoral ciliary zone of *Entodinium* Stein (Ciliophora, Entodiniomorphida). *J. Protozool.,* **30**: 466–472.

8. Latteur, B., Tuffrau, M. & Wespes, G. 1970. *Triplumaria selenica* n. sp., cilié spirotriche du colon de l'éléphant d'Afrique. *Protistologica,* **6**: 319–330.

9. Lubinsky, G. 1958. Ophryoscolecidae (Ciliata: Entodiniomorphida) of reindeer (*Rangifer tarandus* L.) from the Canadian arctic. I. Entodiniinae. *Can. J. Zool.,* **36**: 819–835.

10. Noirot-Timothée, C. 1960. Etude d'une famille de ciliés: les Ophryoscolecidae. Structure et ultrastructures. *Ann. Sci. Nat. Zool., Ser. 12,* **2**: 527–718.

11. Ogimoto, K. & Imai, S. 1981. *Atlas of Rumen Microbiology.* Japan Scientific Societies Press, Tokyo.

12. Párducz, B. 1967. Ciliary movement and coordination in ciliates. *Int. Rev. Cytol.,* **21**: 91–128.

13. Strelkow, A. 1929. Morphologische Studien über oligotriche Infusorien aus dem Darme des Pferdes. 1. Außere Morphologie und Skelett der Gattung *Cycloposthium* Bundle. *Arch. Protistenkd.,* **68**: 503–554.

14. Van Hoven, W., Gilchrist, F. M. C. & Hamilton-Attwell, V. L. 1987. Intestinal ciliated protozoa of African rhinoceros: two new genera and five new species from the white rhino (*Ceratotherium simum* Burchell, 1817). *J. Protozool.,* **34**: 338–342.

15. Wolska, M. 1965. Remarks on the adoral ciliature in the order Entodiniomorpha. *Acta Protozool.,* **3**: 321–324.

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Improved Method for High-Yield Excystation and Purification of Infective Sporozoites of *Eimeria* spp.[1]

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ABSTRACT. This report describes a new, gentle procedure for rapid and efficient excystation of large numbers of infective sporozoites of *Eimeria vermiformis* and *Eimeria stiedai*. Excysted sporozoites are purified using modifications of a previously described ion-exchange chromatography method. The procedure avoids physical breakage of oocysts and results in greater than 70% recovery of the sporozoites present as sporulated oocysts (i.e. 5–6 sporozoites per sporulated oocyst). The recovered sporozoites are greater than 95% pure and are infective in vivo. We routinely isolate greater than 2×10^8 sporozoites without the use of specialized or expensive equipment.

PARASITE recognition and invasion of host tissue by coccidian parasites of the genus *Eimeria* display remarkable species and cell specificity (1, 10, 11, 17). Biochemical studies aimed at isolation of biologically active parasite surface receptors responsible for specific target cell recognition require large amounts of pure sporozoites which are representative of the majority of naturally occurring infectious forms, i.e. those in sporulated oocysts.

There are several reports describing oocyst excystation and sporozoite purification procedures (2, 14, 15, 18, 20) but none report high yields of infective sporozoites based on the starting number of sporulated oocysts. Furthermore, our attempts to use these procedures resulted in an average yield of only 1–1.5 sporozoites per sporulated oocyst.

Purification procedures resulting in such low recoveries do not insure the isolation of sporozoites which are representative of those produced in infected animals. Therefore, the goal of our study was to maximize the yield of sporozoites during in vitro excystation to assure isolation of a quantitatively representative sporozoite population for subsequent biochemical studies.

MATERIALS AND METHODS

Preparation of oocysts. A clone of *E. vermiformis* oocysts was originally established and used to inoculate mice, *Mus musculus* (a coccidia-free colony of outbred Swiss Webster mice maintained at the University of Illinois, College of Veterinary Medicine, was used throughout these studies) as previously described

[1] This work was supported by a Biomedical Research Support Grant from the National Institutes of Health.

[2] This work is in partial fulfillment for the degree Master of Science in the Department of Pathobiology, College of Veterinary Medicine.

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(17). Fecal material was collected from infected mice in 2.5% (w/v) potassium dichromate solution. The oocysts were separated from the majority of fecal debris by filtration through a 150-μm mesh metal screen (U.S. Standard Sieve Series, No. 100, The W. S. Tyler Co., Mentor, OH), concentrated by unit gravity sedimentation, and allowed to sporulate at room temperature.

Initial purification of sporulated oocysts. Sporulated oocysts were concentrated by sedimentation at unit gravity and the 2.5% dichromate removed by decanting. The concentrated oocysts were further purified by sugar flotation as previously described (3).and then washed 3× with water by centrifugation (1000 *g*, 5 min).

The oocyst pellet was resuspended in 15 ml of 5.25% (w/v) sodium hypochlorite and allowed to stand at 4°C for 10 min. The oocysts were then washed 2× with water by centrifugation as described above. These washed oocysts were further cleaned by salt flotation using 45 ml of 20% (w/v) NaCl and centrifugation at 400 *g*, 5 min. Following this centrifugation the cleaned oocysts were diluted at least three-fold with water and washed 3× by centrifugation (1000 *g*, 5 min). The final, cleaned oocysts were resuspended in a minimal volume of 2.5% (w/v) dichromate and stored at 4°C until needed.

Eimeria stiedai oocysts were obtained from liver homogenates of inoculated white rabbits, sporulated, and cleaned as described above for *E. vermiformis*.

Preparation of excystation solutions. The two excystation solutions used were prepared in 200-ml lots and frozen in 10-ml aliquots until needed. Solution A consisted of 0.25% (w/v) crude trypsin (trypsin, 1-250 from hog pancreas extract, ICN Biomedicals, Inc., Costa Mesa, CA) and 0.25% (w/v) taurodeoxycholate (Sigma Chemical Co., St. Louis, MO) dissolved in HAM F-10 medium (GIBCO Laboratories, Grand Island, NY). Solution B contained 0.25% (w/v) taurodeoxycholate in HAM F-10 medium. The pH of both solutions A and B was adjusted to 7.2.

Excystation of oocysts. Oocyst concentration and percent sporulation was determined by direct microscopic counting using a hemacytometer. Approximately 1×10^8 sporulated oocysts were repeatedly washed with distilled water by centrifugation (1000 *g*, 5 min) until the brown color due to potassium dichromate disappeared. To the final washed oocyst pellet, 15 ml of 5.25% (w/v) sodium hypochlorite was added and the oocysts allowed to sit for 24 h at 4°C. The oocysts were then washed twice with water as described above. To the oocyst pellet, 10 ml of excystation solution A was added and the contents transferred to a 25-ml Erlenmeyer flask, placed into a 41°C orbital shaking water bath (200 rpm), and incubated for 1 h for *E. vermiformis* or 2 h for *E. stiedai*.

At the end of the incubation the mixture of oocysts and partially excysted sporozoites were washed twice with cold (4°C) phosphate-buffered saline solution (PBS), pH 7.35, by centrifugation (1000 *g*, 10 min). The pellets were resuspended in 10 ml of solution B and incubated at 41°C for 30 min (for both *E. vermiformis* and *E. stiedai*) as described above. Following this second incubation, the crude sporozoites were washed in PBS as described above and held on ice until further purified by chromatography as described below.

Purification of sporozoites. Anion exchange chromatography of excysted sporozoites was performed using minor modifications of the procedure described by Schmatz et al. (14). Briefly, a column (1 × 3 cm) of DEAE cellulose (DE-52, Whatman Chemical Separation Inc., Clifton, NJ) was prepared from defined resin and equilibrated in 10 column volumes of PBS at a flow rate of approximately 10 ml/min. The crude sporozoite pellet was resuspended in 10 ml of PBS and gently layered over the column. The column was continuously eluted with PBS at a flow rate of 1 ml/min. It was necessary to stir occasionally but carefully the accumulated debris on top of the column resin to maintain adequate flow rate and to free small amounts of trapped sporozoites. A total of 150 ml of eluant was collected from the column. The purified sporozoites were concentrated by centrifugation (1000 *g*, 15 min), resuspended in a minimal volume of HAMS F-10 medium, and stored on ice or frozen (−20°C) until use.

Determination of sporozoite yield and purity. In all experiments, the yield of sporozoites was determined microscopically using a hemacytometer (assuming eight sporozoites per sporulated oocyst). The final purity of the sporozoites was estimated by counting both intact motile sporozoites as well as any other recognizable particle or piece of debris.

Determination of sporozoite cell volume. Determination of sporozoite cell size (cell volume) and estimation of the amount of contaminating debris derived from oocyst shells and sporocyst fragments was done by electronic particle counting using a Coulter Counter model ZM. Aliquots of the final purified sporozoites were placed in a final volume of 100 ml of Isotone (Coulter Electronics, Hialeah, FL) to achieve a final cell concentration of $7.5–8.0 \times 10^4$ cells/ml. The 100-ml suspension was gently stirred throughout the sizing measurements. The Coulter Counter was first calibrated with 4.98-μm diameter latex beads. The settings were as follows: amplification (A) = 8, aperture current (I) = 300 mAmps, matching switch = 10 kohms, and the full scale switch = 10 mAmps.

RESULTS

Cleaning of oocysts. The cleaned oocysts (Fig. 1B) were almost entirely free of fecal debris (Fig. 1A); however, occasional contamination with *Scyphacia obvoleta* ova and fungal mycelia was observed. These contaminants were easily removed during the final steps of sporozoite purification.

Eimeria stiedai oocysts could also be separated from much of the contaminating liver tissue and debris by using the same cleaning procedure developed for *E. vermiformis*. The initial 10-min incubation in sodium hypochlorite was effective in removing some of this contamination. It was noted, however, that during flotation in 20% (w/v) NaCl, much of the immature and unsporulated oocysts and remaining liver tissue floated to the top whereas the sporulated oocysts remained at the bottom of the NaCl. Following the final 24-h bath in sodium hypochlorite and subsequent washing steps performed just prior to excystation, greater than 98% of the liver debris and immature oocysts had been removed.

Excystation of oocysts (effect of temperature). There was only a gradual increase in the percentage of sporozoites released from

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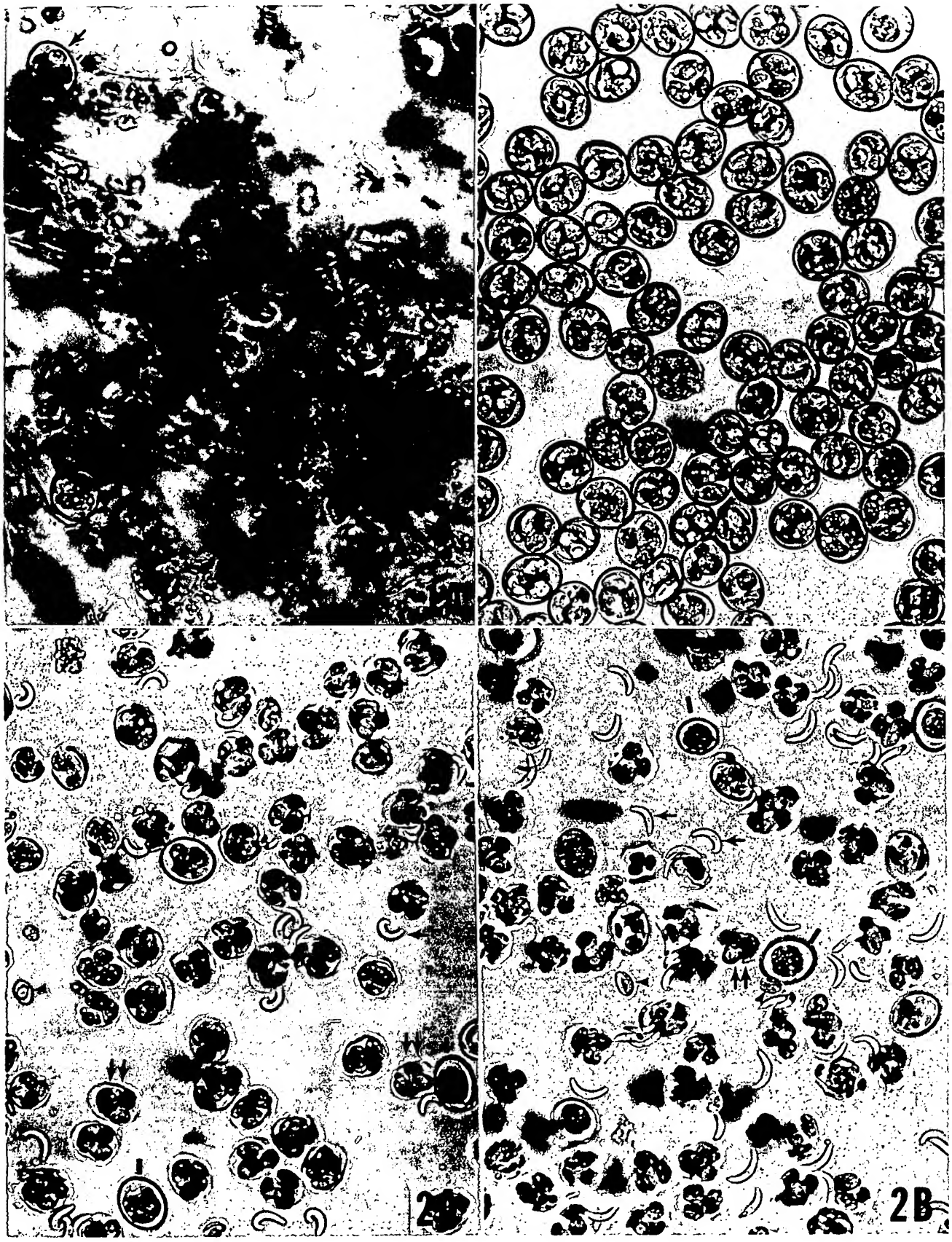
Figs. 1, 2. Brightfield photomicrographs of crude and cleaned *E. vermiformis* oocysts and oocysts in the process of excystation. Crude oocysts were collected and cleaned from fecal debris as described in Materials and Methods. 1A. Crude oocysts, arrow indicates a sporulated oocyst; 1B. Cleaned oocysts. ×2600. 2. *Eimeria vermiformis* oocysts were incubated at 41°C in the presence of both trypsin and taurodeoxycholate as described in Materials and Methods and Fig. 3. Brightfield photomicrographs were taken at the following times during excystation: A. 30 min; B. 60 min. The arrows indicate excysted sporozoites. Empty sporocysts are denoted by arrowheads. Double arrows point to oocysts which display thinned walls as compared to the thick walls of unsporulated oocysts (dash). ×2600.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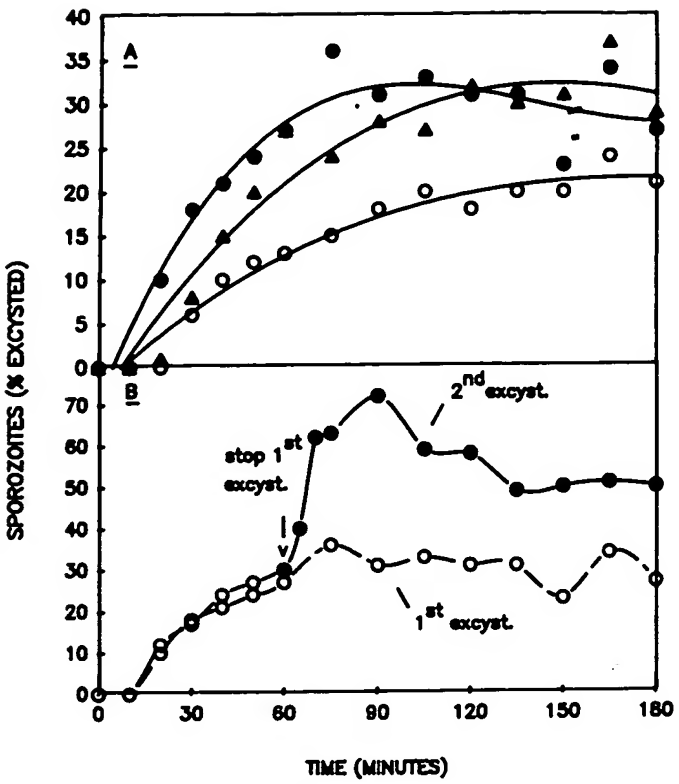


Fig. 3. Kinetics of *E. vermiformis* excystation. *Eimeria vermiformis* sporulated oocysts were cleaned, treated with or without sodium hypochlorite at 4°C for 24 h, and excysted at 41°C for the indicated times in the presence of both trypsin and taurodeoxycholate or taurodeoxycholate alone as described in Materials and Methods. A. One-step incubation; (O—O), oocysts treated in the absence of sodium hypochlorite and excysted in the presence of both trypsin and taurodeoxycholate; (●—●), oocysts treated in the presence of sodium hypochlorite and excysted in the presence of both trypsin and taurodeoxycholate; (▲—▲), oocysts treated in the presence of sodium hypochlorite and excysted in the presence of only taurodeoxycholate. B. Comparison of one- and two-step excystation incubations; (O—O), oocysts were treated with sodium hypochlorite and excysted in a one-step incubation in the presence of both trypsin and taurodeoxycholate; (●—●), oocysts treated with sodium hypochlorite and excysted in a two-step incubation with trypsin and taurodeoxycholate for 1 h and then for the remaining time in the presence of only taurodeoxycholate as described in Materials and Methods.

both *E. stiedai* and *E. vermiformis* when the temperature was raised from 37 to 39°C. The percent release rose dramatically to a sharp maximum, however, when the temperature was raised to 41°C and was followed by a pronounced decrease in the numbers of viable sporozoites recovered at 42–44°C (data not shown). These results indicate that as little as a 1°C variation from 41°C significantly lowers the number of sporozoites excysted.

Kinetics of excystation (effect of sodium hypochlorite pretreatment and a two-step incubation). The effects of sodium hypochlorite pretreatment and a two-step incubation on the excystation kinetics of *E. vermiformis* are given in Fig. 3A and B, respectively. Pretreatment of the cleaned, sporulated oocysts with sodium hypochlorite for 24 h at 4°C markedly increased both the rate and extent of sporozoites released during the excystation incubation (Fig. 3A). This effect occurred regardless of whether the incubation was conducted in the presence of both trypsin and taurodeoxycholate or taurodeoxycholate alone. As expected, the rate of excystation of pretreated oocysts was greatest when both trypsin and taurodeoxycholate were included in the incubation; however, it was surprising that the excystation rate was only slightly reduced when the incubation was performed in the absence of trypsin (Fig. 3A).

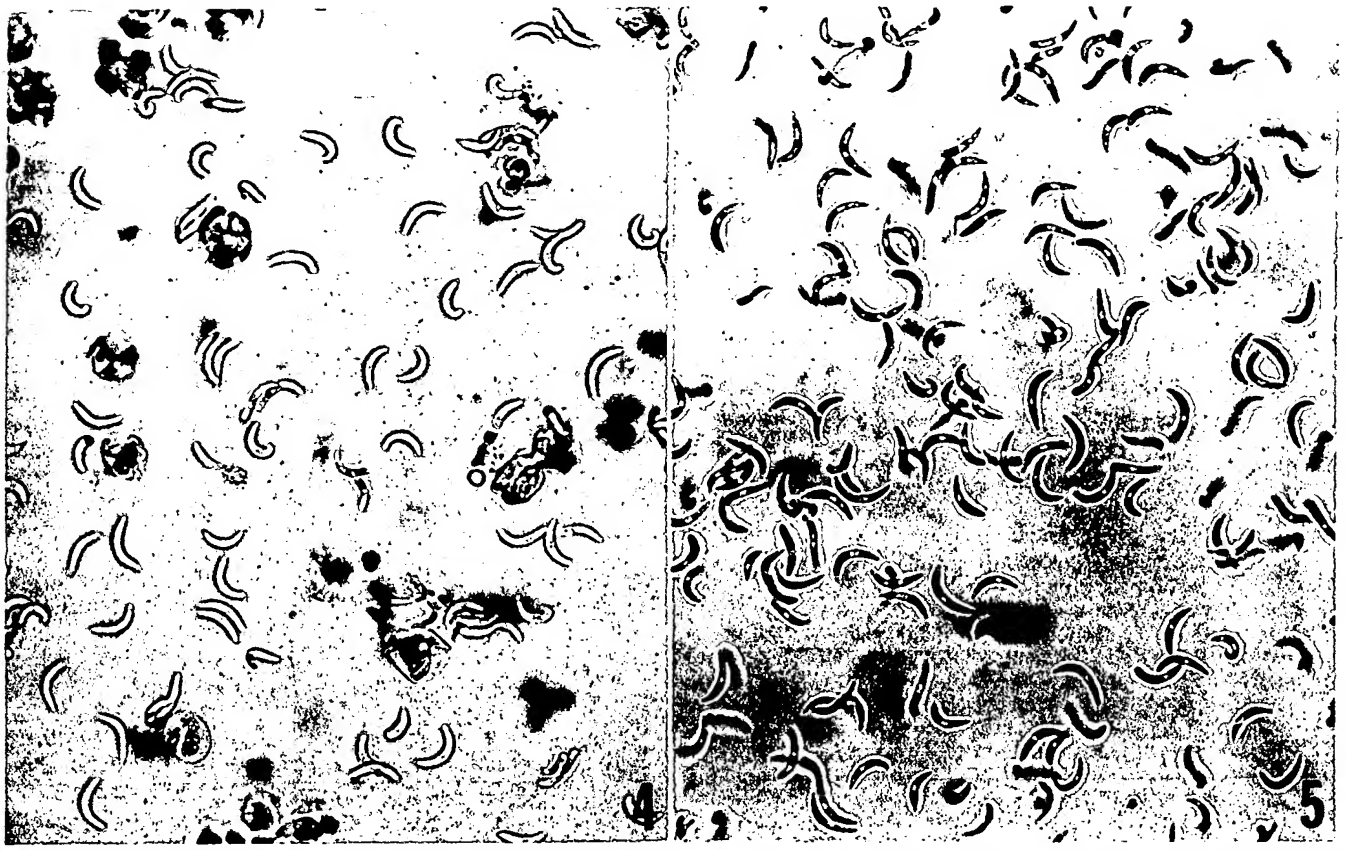
When oocysts pre-treated with sodium hypochlorite were incubated in the presence of both taurodeoxycholate and trypsin there was an initial 20-min lag, followed by a rapid increase in the number of released sporozoites. At approximately 60 min, the number of excysted sporozoites reached a plateau value of about 30–35% of the theoretical number of starting sporozoites (Fig. 3A). Although the plateau concentration of released sporozoites was maintained for an additional 60 min of incubation, the percent viability, as measured by trypan blue staining, decreased by 10–20%. In addition, about 10–15% of the sporozoites which did not stain by trypan blue contained cell surface blebs and/or a more granulated and less refractive appearance. During subsequent purification of these sporozoites incubated for a long term, the recovery of viable cells was consistently lower than would have been predicted by the percentage released during excystation. These results suggested the decline in sporozoite recovery was due to destruction of released sporozoites from prolonged exposure to the excystation solution.

Based on the above observation and the fact that excystation could occur in the presence of only taurodeoxycholate, we attempted to maximize the release of sporozoites while simultaneously limiting their exposure to the caustic excystation solutions. Our most successful experiments resulted from using a two-step excystation procedure. When the first incubation (trypsin plus taurodeoxycholate) was performed for only 60 min, we eliminated most of the sporozoite destruction. As shown in Fig. 2, however, it was obvious that optimum release of sporozoites had not occurred by the end of this incubation. Only about 30–35% of the total sporozoites had been excysted. In an effort to increase sporozoite yield several additional incubations were tried. The best second excystation incubation, which stimulated the release of another 30–35% of the starting sporozoites, was a 30-min exposure to taurodeoxycholate in the absence of trypsin (Fig. 3B). The high percentage of free sporozoites and the relative absence of intact sporulated oocysts found at the end of this incubation (Fig. 4) indicated that most of the sporulated oocysts had excysted. Thus, by prematurely stopping the initial exposure to trypsin and taurodeoxycholate followed by a second shorter incubation in only taurodeoxycholate, we achieved maximum release of sporozoites without the concomitant destruction that occurred in single long-term incubations.

The kinetics of excystation during the second incubation indicated maximum release of sporozoites after 30 min. This maximum was followed by a rapid decline in the percentage of recovered sporozoites after 40 to 180 min (Fig. 3B). Therefore, it is important that this second incubation be conducted precisely for 30 min at 41°C.

The excystation kinetics obtained for *E. stiedai* were somewhat different from those of *E. vermiformis*. When oocysts were pre-treated for 24 h in sodium hypochlorite and then incubated in trypsin and taurodeoxycholate, there was a 60–70-min lag before any release of sporozoites was observed. Following the lag, excystation proceeded linearly for 60 min (Fig. 6).

We were able to maximize the yield of viable sporozoites using the same strategy as described above. The first excystation incubation was stopped after 120 min, the oocysts washed and placed in taurodeoxycholate for an additional 30 min (see Fig. 6 and Materials and Methods). As was described above for *E. vermiformis*, the second incubation in taurodeoxycholate must be done precisely for 30 min if optimal recoveries of excysted sporozoites are to be achieved.



Figs. 4, 5. Brightfield photomicrograph of *E. vermiformis* oocysts at the end of the two-step excystation incubation and phase contrast micrograph of purified sporozoites. 4. The oocysts were subjected to a two-step excystation incubation as described in Materials and Methods. At the end of the 30-min second incubation, an aliquot of the suspension of oocysts was photographed under brightfield microscopy. 5. Phase contrast micrograph of sporozoites obtained following the second excystation incubation and purified by ion exchange chromatography as described in Materials and Methods. ×2600.

We also observed, in both species, a marked thinning of the sporulated oocyst walls near the end of the lag phase during incubation in trypsin and taurodeoxycholate. This thinning appeared to be specific for sporulated oocysts (double arrows, Fig. 2).

The percentages of released sporozoites following the two excystation incubations for both *E. vermiformis* and *E. stiedai* was markedly higher than has been previously reported (7, M. Elaine Rose, pers. commun.). We have achieved recoveries of viable sporozoites of greater than 80% of the theoretical number present in sporulated oocysts. Typical recoveries average greater than 70% for both species.

Purification of sporozoites. Of the various sporozoite purification methods we have tried, we found the anion exchange chromatography method of Schmatz et al. (14) to be the most rapid and efficient technique for purification of large numbers of sporozoites. A single small column (1 × 3 cm) was easily able to purify 1 × 10^9 sporozoites. Typically, greater than 85% of the loaded sporozoites could be recovered from the column after elution in PBS as described in Materials and Methods. Elution of the column for longer than 2 h should be avoided since it resulted in increased contamination with excystation debris. Following chromatography, the sporozoites were separated from the fine debris particles by differential centrifugation (see Materials and Methods) and evaluated for degree of purity as described below.

The purified sporozoites were nearly homogeneous as judged by light microscopy (Fig. 5) and cell-sizing measurements (Fig.

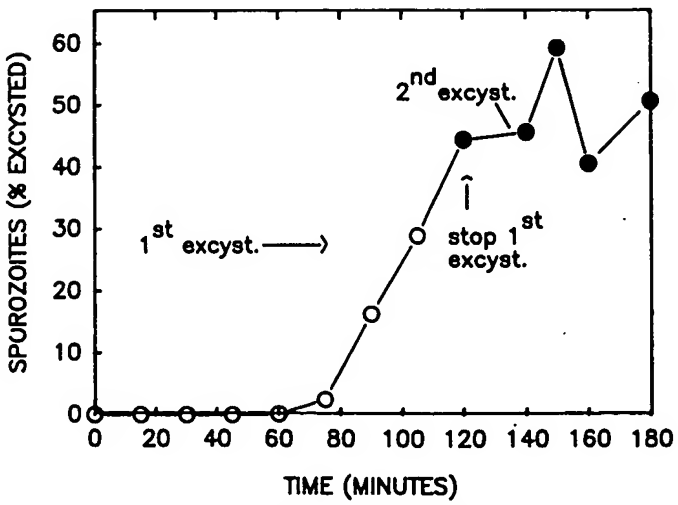


Fig. 6. Kinetics of *E. stiedai* excystation. *Eimeria stiedai* oocysts were prepared, cleaned, and subjected to the two-step excystation incubation as described in Materials and Methods. The kinetics of the first (O—O) and second (●—●) excystation incubations are denoted separately on the figure. Note the first incubation was for 120 min for *E. stiedai* oocysts as compared to 60 min for *E. vermiformis* oocysts (Fig. 3B).

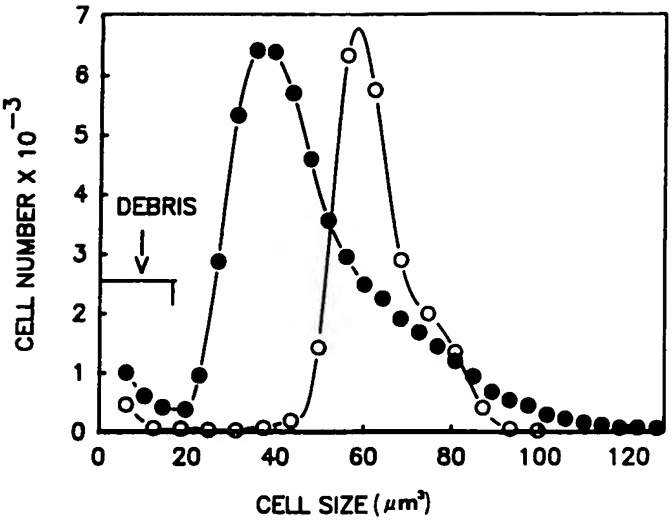


Fig. 7. Particle volume profile of purified *E. vermiformis* sporozoites. The degree of cell size (volume) homogeneity and small particle debris contamination of purified *E. vermiformis* sporozoites was estimated by particle-sizing analysis using a Coulter electronic counter as described in Materials and Methods. (O—O), 4.98-μm-diameter standard latex particle; (●—●), purified *E. vermiformis* sporozoites.

7). The sporozoites are entirely free of contaminating oocyst shells, broken sporocysts, or other large debris. By counting both sporozoites and debris, we estimated the final sporozoite purity was routinely greater than 96%.

The same degree of purity was obtained by determining the average particle size of the *E. vermiformis* sporozoite population using an electronic particle counter as described in Materials and Methods. The counter allows separate quantification of the number of particles in a population based on differences in particle or cell volume. The majority of sporozoites displayed a near symmetrical peak ranging in volume from 24 to 60 μm^3 with the average sporozoite being about 40 μm^3 (Fig. 7). Microscopy showed some single sporozoites which appeared substantially larger than most others as well as a few clumps of two and three sporozoites. These cells and aggregates are most likely responsible for the larger volume (60–90 μm^3) particles seen as a tail on the main peak. Only very small amounts of debris (1–20 μm^3 particle volume) were detected in the pure *E. vermiformis* sporozoite preparation. The number of particles in the small debris fraction is less than 5% and agrees well with the value of 96% purity estimated by microscopy. A summary of the yields of viable sporozoites obtained at each step of the purification is shown in Table I.

TABLE I. *Summary of the excystation and purification of sporozoites.*[a]

Purification step	Cell number × 10^6	
	E. vermiformis	*E. steidai*
Sporulated oocysts	100	8.0
Sporozoites (theoretical)	800	64.0
1st excystation	280 (35%)[b]	28.4 (44%)
2nd excystation	600 (75%)	38.4 (60%)
DEAE chromatography	560 (70%)	36.4 (57%)
Purity	>95%	>95%

[a] The final purity was estimated by light microscopy and electronic particle counting as described in Materials and Methods.
[b] The numbers in parentheses indicate the percent yield at each step.

TABLE II. *Infectivity of purified* E. vermiformis *sporozoites.*

Sample inoculated[a]	Oocysts/g feces × 10^6 [b]
Control (no oocysts)	0.0
12,500 oocysts (P.O.)	30.2
100,000 sporozoites (P.O.)	24.8
12,500 oocysts (I.P.)	1.0
100,000 sporozoites (I.P.)	1.0

[a] The number of oocysts/g feces was determined by microscopy.
[b] Oocysts or sporozoites were inoculated into coccidia-free mice by either intraperitoneal (I.P.) or per os (P.O.) injection.

Infectivity of purified E. vermiformis *sporozoites.* The final preparation of purified sporozoites was infective when inoculated into mice (Table II). The purified sporozoites were quantitatively as infective as an equivalent number of crude sporozoites injected as sporulated oocysts rather than sporozoites. Furthermore, essentially identical numbers of oocysts were obtained per gram of feces from inoculated mice regardless of whether the sporozoites or sporulated oocysts were injected per os or intraperitoneally. These results indicate that the large scale excystation and purification procedure described in this report yields sporozoites which are as infective for mice as the crude oocysts from which they were purified.

DISCUSSION

The molecular mechanisms involved in excystation of eimerian oocysts are not well understood. Current published procedures for in vitro excystation neither claim to have obtained maximum yield of sporozoites nor do they report yields calculated from the starting number of sporulated oocysts (2, 8, 15, 18, 20). Therefore, the criteria we used in the development of an efficient in vitro excystation procedure were 1) it should use conditions which resemble the in vivo environment, 2) it should be relatively rapid and not require specialized or expensive equipment, and 3) it must result in excystation of the majority of sporozoites present in the starting sporulated oocysts.

In this study we have monitored the kinetics of excystation under various incubation conditions in an attempt to preserve sporozoites as well as to excyst as many sporulated oocysts as possible. Using this approach, we have developed a relatively rapid, simple procedure which yields greater than 1×10^9 purified sporozoites in 4–6 h following the 24-h sodium hypochlorite pretreatment step. The excystation conditions resemble those likely to exist in vivo and do not involve physical breakage of oocysts. The recovered sporozoites are greater than 96% pure and are as infective for mice as an equivalent number of sporozoites inoculated as sporulated oocysts.

The method of sporozoite excystation and purification described here is the first procedure that routinely yields greater than 70% of the theoretical number of sporozoites present in the sporulated oocysts. The percentage of sporozoites obtained is probably underestimated because the theoretical number is calculated based on a microscopic count of sporulated oocysts which may include some immature sporulated oocysts that are not yet ready to excyst. The sporozoite recoveries we report are 3–7-fold greater than those obtained in previous procedures (8, M. Elaine Rose, pers. commun.). Most importantly, since the procedure allows isolation of the majority of sporozoites contained within sporulated oocysts, it insures isolation of a biologically representative cell population that can be used for biochemical studies.

The use of two flotation steps (flotation first in Sheather's solution and then in 20% [w/v] NaCl separated by a 10-min incubation in sodium hypochlorite) greatly facilitates the re-

moval of most of the fecal debris. Furthermore, the cleaning and excystation procedures used here allowed us to avoid the physical breaking of oocysts in a tissue homogenizer (9, 14, 15, 20). Consequently, our preparations contained less debris and fewer damaged sporozoites as compared to preparations made by using homogenization steps.

The 24-h incubation in sodium hypochlorite increased the sporozoite yield by 1.5- to 2-fold with an additional 50–100% increase arising from the inclusion of the second excystation incubation. The sodium hypochlorite treatment also eliminated the need for carbon dioxide or reducing agents which have been reported to be necessary for adequate excystation of sporulated *E. stiedai* oocysts in vitro (5, 9).

During excystation of both species we observed a definite thinning of oocyst cell walls which appeared specific for sporulated oocysts. The thinning occurred within 10 min of the first excystation incubation for *E. vermiformis* and at about 1 h for *E. stiedai*. A similar change in morphology has been previously reported to occur during excystation in the presence of L-cysteine (12).

The specificity of the wall thinning for sporulated oocysts suggests that the oocyst wall may be modified during sporulation. Such a modification could occur by a variety of mechanisms (6, 7, 13, 16, for review see 19). Albeit conjecture, one potentially interesting mechanism is the induction of one or more sporozoite-specific wall modification enzymes. One possible candidate for such an enzyme is sporozoite acid phosphatase. We have recently found that this enzyme activity dramatically increases during sporulation of *Eimeria* spp. and is present in higher specific activity than any other hydrolytic enzyme we have measured (4). We are currently investigating the role, if any, that acid phosphatase may play in sporozoite excystation and subsequent host cell interaction.

LITERATURE CITED

1. Adams, J. H. & Todd, K. S., Jr. 1983. Transmission electron microscopy of intracellular sporozoites of *E. vermiformis* (Apicomplexa, Eucoccidiida) in the mouse. *J. Protozool.*, **30**: 114–118.

2. Bontemps, M. & Yvore, P. 1974. Technique de purification des suspensions de sporozoites d'*Eimeria* sur colonne de fibres synthetiques. *Ann. Rech. Vet.*, **5**: 109–113.

3. Hammond, D. M., Chobotar, B. & Ernst, J. V. 1968. Cytological observations on sporozoites of *Eimeria bovis* and *Eimeria auburnensis* and an eimerian species from the Ord kangaroo rat. *J. Parasitol.*, **54**: 550–558.

4. Hosek, J. E., Todd, K. S., Jr. & Kuhlenschmidt, M. S. 1988. Demonstration of acid phosphatase in *Eimeria* spp.: partial characterization of the enzyme of *E. vermiformis*. *J. Protozool.*, **35**: 531–532.

5. Jensen, J. B., Nyberg, P. A., Burton, S. D. & Jolley, W. R. 1976. The effects of selected gases on excystation of coccidian oocysts. *J. Parasitol.*, **62**: 195–198.

6. Jolley, W. R., Allen, J. V. & Nyberg, P. A. 1979. Micropyle and oocyst wall changes associated with chemically mediated in vitro excystation of *Eimeria stiedai* and *Eimeria tenella*. *Int. J. Parasitol.*, **9**: 199–204.

7. Jolley, W. R., Burton, S. D., Nyberg, P. A. & Jensen, J. B. 1976. Formation of sulfhydryl groups in the walls of *Eimeria stiedai* and *Eimeria tenella* oocysts subjected to in vitro excystation. *J. Parasitol.*, **62**: 199–202.

8. Larsen, R. A., Kyle, J. E., Whitmire, W. M. & Speer, C. A. 1984. Effects of nylon wool purification on infectivity and antigenicity of *Eimeria falciformis* sporozoites and merozoites. *J. Parasitol.*, **70**: 597–601.

9. Nyberg, P. A., Bauer, D. H. & Knapp, S. E. 1968. Carbon dioxide as the initial stimulus for excystation of *Eimeria tenella* oocysts. *J. Protozool.*, **15**: 144–148.

10. Rose, M. E. & Millard, B. J. 1985. Host specificity in eimerian coccidia: development of *E. vermiformis* of the mouse, *Mus musculus*, in *Rattus norvegicus*. *Parasitology*, **90**: 557–563.

11. Rose, M. E., Owen, D. G. & Hasketh, P. 1984. Susceptibility of coccidiosis: effect of strain of mouse on reproduction of *E. vermiformis*. *Parasitology*, **88**: 45–54.

12. Ryley, J. F. 1973. Cytochemistry, physiology, and biochemistry, *in* Hammond, D. M. & Long, P. L., eds., *The Coccidia:* Eimeria, Isospora, Toxoplasma *and Related Genera*. University Park Press, Baltimore, pp. 145–181.

13. Ryley, J. F. 1980. Recent developments in coccidian biology; where do we go from here? *Parasitology*, **80**: 189–209.

14. Schmatz, D. M., Crane, M. St. J. & Murray, K. P. 1984. Purification of *Eimeria* sporozoites by DE-52 anion exchange chromatography. *J. Protozool.*, **31**: 181–183.

15. Stotish, R. L., Simashkevich, P. M. & Wang, P. M. 1977. Separation of sporozoites, sporocysts, & oocysts of *Eimeria tenella* by centrifugal elutriation. *J. Parasitol.*, **63**: 1124–1126.

16. Stotish, R. L., Wang, C. C. & Meyenhofer, M. 1978. Structure and composition of the oocyst wall of *Eimeria tenella*. *J. Parasitol.*, **64**: 1074–1081.

17. Todd, K. S., Jr. & Lepp, D. L. 1971. The life cycle of *E. vermiformis* Ernst, Chobotar and Hammond, 1971 in the mouse *Mus musculus*. *J. Protozool.*, **18**: 332–337.

18. Wagenbach, G. E. 1969. Purification of *Eimeria tenella* sporozoites with glass bead columns. *J. Parasitol.*, **55**: 833–834.

19. Wang, C. C. 1982. Biochemistry and physiology of coccidia, *in* Long, P. L., ed., *The Biology of the Coccidia*. University Park Press, Baltimore, pp. 181–185.

20. Wisher, M. H. & Rose, M. E. 1984. The large-scale preparation of purified sporozoites of *Eimeria* spp. by metrizamide density-gradient centrifugation. *Parasitology*, **88**: 515–519.

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